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VÝVOJ ELEKTROCHEMICKÉHO GLUTAMÁTOVÉHO BIOSENZORU

Diplomová práce

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DEVELOPMENT OF A GLUTAMATE ELECTROCHEMICAL BIOSENSOR

Diploma Thesis

in cooperation with
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CONTENTS

1 INTRODUCTION

We know sensations very well from our daily life. Our nose, tongue, ears, eyes and fingers, together with specific areas of the brain, are ”sensors“ we use every day without awareness to perceive the real world around. As their extrapolations in the laboratory context, using of the litmus paper or the pH meter is so common in the same way. However, these mentioned sensors are not the only examples. We can find many types of them in all areas related with human activities for detecting all kinds of energy as thermal, electrical, mechanical, optical, acoustic, etc.

In order that a chemical sensor could work well, it is necessary to choose a recognising element able to select and monitor the target chemical property. Using enzymes is very suitable for this goal, since we can take advantage of their main features such as high selectivity and sensitivity towards a given substrate. In this report, the role of the substrate is presented by L-glutamate which is catalytically converted by L-glutamate dehydrogenase.

L-glutamic acid and monosodium glutamate appear now in the field of interest of many scientific groups included medical and food-industry-analytical experts, since these substances have become to be used as a flavour enhancer in the most of industrially prepared food. Although glutamate is normally a harmless substance, as a component of all natural proteins and a participant of many metabolic processes, some people have experienced adverse effects, such as stiffness and tension, after its ingestion. Besides, some studies demonstrated its connection with the appearance of neurological manifestations, such as Parkinson´s and Alzheimer´s disease. It is probable that in the future we will need to keep the glutamate content in all food products under certain limits. Thus, large-scale applicable, fast and economic methods of L-glutamic acid and L-glutamate determination would be very appreciated. Determination by an electrochemical biosensor could be one of these methods.

1.1 AIM OF THE WORK

This work is concerned with the development of a novel L-glutamate biosensor using a dehydrogenase enzyme. The enzyme is incorporated by entrapment into the surface

layer contacting with the sample, made of an inert ceramic material. To convert enzymatic activity into a measurable signal, electrochemical transduction was selected. Therefore, the surface layer was deposited over a conducting surface that fills a rod shaped Teflon body.

The L-glutamate dehydrogenase requires nicotinamide ($NAD(P)^+$/NAD(P)H) coenzyme for its activity. To avoid continuous wasting of the added coenzyme electrochemical regeneration of its oxidised or reduced form could be implemented. However, the need of high potential for direct NAD(P)H oxidation can cause severe interferences by other species present in real samples. In this view, several electrocatalysts for the oxidation of NADPH at lower potential were tested, being the water-soluble dye, methylene green, selected for this purpose. To provide a thin film of methylene green on the graphite electrode surface the method of electropolymerization is applied.

All measuring process is served by voltammetric and amperometric methods with either a batch or flow-injection system.

2 THEORETICAL PART

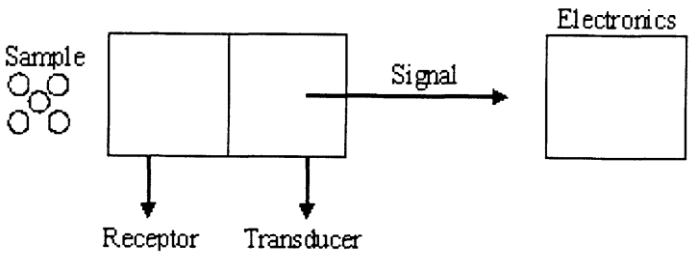
2.1 SENSORS AND BIOSENSORS

A sensor can be defined as a miniaturized device that detects or measures a sample property and records, indicates or otherwise responds to it. We can divide sensors into three types:

- *physical sensors*, for measuring physical quantities such as lenght, weight, temperature, pressure and electricity;
- *chemical sensors*, which measure chemical substances by chemical or physical responses and can be used for the qualitative or quantitative determination of the analyte;
- *biosensors*, which measure chemical substances by using a biological sensing element, which is incorporated and connected to a transducer (see Fig. 1). (More information about the way of *transduction* is following.) Biosensors are sometimes considered as a sub-set of chemical sensors [1].

Although being an example of physical sensor, the thermometer could be used to define the main aspects of the devices classified as sensors. Required specifications of a sensor are its ability to give reversible and continuous information (otherwise called probe), to provide it in real-time, to overpass the need of elaborate sample pre-treatment and to provide remote (out-of-lab) determinations. When considering chemical sensors common examples are more dificult to find simply because most of known chemical reactions are irreversible. Thus, as obvious choice of many sensor developpers as well in the herein reported work attention is given to biosensors.

Figure 1: Schematic diagram of biosensor device [3].



Several types of biological sensing elements can be employed in biosensors, namely enzymes, antibodies, mitochondria, receptors and nucleic acids. The use of whole cells and organic tissue was also reported [1].

The high selectivity, sensitivity and quite easy handling of the enzymes make their use in analysis techniques very profitable. However in aqueous solutions, enzymatic catalytic activity is usually lost rather rapidly, because enzymes can suffer oxidation reactions or its tertiary structure can be destroyed at the air-water interface. Moreover, assays based on reconstituted solutions of enzymes determine their continuous spoil since one knows they kept their catalytic properties at the end.

These aspects make the use of enzyme reagents both expensive and complex. These problems can be minimized by enzyme immobilization. Moreover, the use of enzymes in the form of biosensor seems quite advantageous.

The successful biosensor should possess the following features:

- the biological component must be specific and stable;
- the reaction should be as independent of physical parameters, such as pH, temperature and stirring, as possible;
- the response should be accurate, precise and reproducible;
- the sensing element should be tiny and biocompatible;
- the complete unit should be cheap and portable.

2.1.1 AREAS OF APPLICATION

Sensors can be used in several different areas. Some examples are listed below:

- Health Care (measurements of blood, gases, ions and metabolites, included glucose, urea, lactate, cholesterol, sodium, potassium, calcium, oxygen; measuring of pH; detection of Hepatitis B, Candida albicans, Penicillins);
- Control of Industrial Processes; food and beverage industry can be mentioned as one of many examples (monitoring of reactants and products, such as sugars, yeasts, malts, alcohols, phenolic compounds);
- Enviromental Monitoring (pollution applications, farming, gardening, veterinary science, mining) [1].

2.2 TRANSDUCTION

The transducer can be described as the sensor component that converts the monitored change (physical or chemical) into a measurable signal. In a broader sense, it converts a signal from one form to another. In chemical sensors and biosensors, the signal is usually an electronic signal whose magnitude is proportional to the concentration of a specific chemical or set of chemicals. In our work the way of transduction was carried out by voltammetric and amperometric principles.

2.2.1. CLASSIFICATION OF TRANSDUCERS

Transducers can be subdivided into the following four main types [1]:

❖ *ELECTROCHEMICAL TRANSDUCERS*

- **Potentiometric.** These involve the measurement of the electromotive force (potential) of a cell at null intensity current condition. The electromotorive force is proportional to the logarithm of the concentration of the substance being determined.
- **Voltammetric.** The current intensity produced in the cell is result of chemical reactions driven by an increasing (or decreasing) applied potential. The current intensity is directly proportional to the concentration of the electroactive material if the appropriate oxidation (reduction) potential is known. One may step the potential directly to that value and observe current. This mode is known as **amperometric.**
- **Conductometric.** Reactions involve changes in the electrolyte composition of the solution. This will normally result in a change in the electrical conductivity of the solution.
- **FET-based sensors.** Miniaturization can sometimes be achieved by constructing one of the above types of electrochemical transducers on a silicon-chip-based field-effect transistors.

❖ *OPTICAL TRANSDUCERS*

These have been taken a new lease of life with the development of fibre optics, thus allowing greater flexibility and miniaturization. The techniques used include absorption

spectroscopy, fluorescence spectroscopy, luminescence spectroscopy, internal reflection spectroscopy, surface plasmon spectroscopy and light scattering.

❖ *PIEZO-ELECTRIC DEVICES*

These devices involve the generation of electric currents from a vibrating crystal. The frequency of vibration is affected by the mass of material adsorbed on its surface, which could be related to changes in a reaction. **Surface acoustic wave devices** are a related system.

❖ *THERMAL SENSORS*

All chemical and biochemical processes involve the production or absorption of heat. This heat can be measured by sensitive thermistors and hence be related to the amount of substance to be analysed.

Following tables (Tab. 1 and Tab. 2) summarize types of receptors in biosensors with electrochemical measurement techniques and types of transducers used in biosensors [2].

Table 1: Types of receptors used in biosensors and the electrochemical measurement techniques, linked to them, which recognize specific species. Biological receptors, which are part of electrochemical biosensors, are indicated in bold type.

Analytes	Receptor / chemical recognition system	Measurement technique / transduction element
1. Ions	Mixed valence metal oxides; permselective, ion-conductive inorganic crystals; trapped mobile synthetic or **biological ionophores**; ion exchange glasses; **enzyme(s)**	**Potentiometric, voltammetric**
2. Dissolved gases, vapours, odours	Bilayer lipid or hydrophobic membrane; inert metal electrode; **enzyme(s)**; **antibody, receptor**	In series with 1; **amperometric;** **amperometric or potentiometric**; **amperometric, potentiometric or impedance**, piezoelectric, optical
3. Substrates	**Enzyme(s)**; **whole cells;** **membrane receptors;** **plant or animal tissue**	**Amperometric or potentiometric; in series with 1 or 2 or metal or carbon electrode, conductometric**, piezoelectric, optical, calorimetric; **as above;** **as above;** **as above**
4. Antibody/ antigen	**Antigen/ antibody;**	**Amperometric, potentiometric or**

	oligonucleotide duplex, aptamer; **enzyme labelled;** chemiluminescent or fluorescent labelled;	**impedimetric**, piezoelectric, optical, surface plasmon resonance; **in series with 3**; optical
5. Various proteins and low molecular weight substrates, ions	**Specific ligands** **protein receptors and channels;** **enzyme labelled;** fluorescent labelled	**As 4**
Besides quantification of the above-mentioned analytes, biosensors are also used for detection and quantification of *micro-organism*; receptros are bacteria, yeast or oligonucleotide probes to electrochemical, piezoelectric, optical or calorimetric transducers.		

Table 2: Types of electrochemical transducer for classified types of measurement, with corresponding analytes to be measured

Meaurement type	Transducer	Transducer analyte
1. Potentiometric	Ion-selective electrode (ISE); glass electrode; gas electrode; metal electrode	K^+, Cl^-, Ca^+, F^- H^+, Na^+…; CO_2, NH_3; redox species
2. Amperometric	Metal or carbon electrode; chemically modified electrodes (CME)	O_2, sugars, alcohols…; sugars, alcohols, phenols, oligonucleotides…
3. Conductometric, impedimetric	Interdigitated electrodes; metal electrode	Urea, charged species, oligonucleotides…
4. Ion charge or field effect	Ion-sensitive field-effect transistor (ISFET); enzyme FET (ENFET)	H^+, K^+…
Non-electrochemical transducers are also used within biosensors: (a) *piezoelectric* (shear and surface acoustic wave); (b) *calorimetric* (thermistors); (c) *optical* (planar wave guide, fibre optic, surface plasmon resonance…).		

2.3 CONSTRUCTION OF BIOSENSORS

2.3.1 IMMOBILIZATION OF BIOLOGICAL COMPONENTS

One important issue related with the biosensor stability refers to the need of the biological component which has to be properly attached to the transducer. This process is known as *immobilization*. There are five main methods of doing this, as follows [1].

❖ *ADSORPTION*

This is the simplest approach and involves minimal preparation. Many substances adsorb enzymes on their surfaces, e.g. alumina, charcoal, clay, cellulose, kaolin, silica gel, glass and collagen. No reagents are required, there is no clean-up step and there is less disruption to the enzymes.

Adsorption can roughly be divided into two forms, namely physical adsorption (*physisorption*) and chemical adsorption (*chemisorption*). Physisorption is usually weak and occurs via the formation of van der Waals bonds, occasionally including hydrogen bonds or charge-transfer forces. Chemisorption is much stronger and involves the formation of covalent bonds.

Adsorbed biomaterial is very susceptible to changes in pH, temperature, ionic strength and the substrate. The bonding is weak and this method is only suitable for exploratory work over a short time-span.

❖ *MICROENCAPSULATION*

This was the method used in the early biosensors. It was developed for the first glucose biosensor on the oxygen electrode. In this technique, an inert membrane, e.g. cellulose acetate (dialysis membrane), is used to trap the biomaterial on to the transducer, thus giving close contact between them. Such a method is adaptable, does not interfere with the reliability of the enzyme, and limits contamination and biodegradation. It is also stable towards changes in pH, temperature, ionic strength and chemical composition. However, such system may be permeable to some materials, e.g. small molecules, including gases, and electrons.

❖ *ENTRAPMENT*

In this approach, the biomaterial is mixed with a monomer solution, which is then polymerized to a gel, thus trapping the material. Unfortunately, this can cause barriers to the diffusion of substrate, thus slowing the reaction and the response time of the sensor. It can also result in loss of bioactivity through pores in the gel – this effect can be counteracted however, by cross-linking. The most commonly used gel is polyacrylamide, furthermore starch, nylon and silastic gels and conducting polymers (such as polypyrrole).

❖ *CROSS-LINKING*

Here, the biomaterial is chemically bonded to solid supports or to another supporting material, such as a gel. Bifunctional reagents, e.g. glutaraldehyde, can be used in such techniques. Again, there is some diffusion limitation and there can also be damage to the biomaterial. In addition, the mechanical strength of the system is poor, because of low rigidity. Nevertheless, it can be a useful method for stabilizing adsorbed biomaterials.

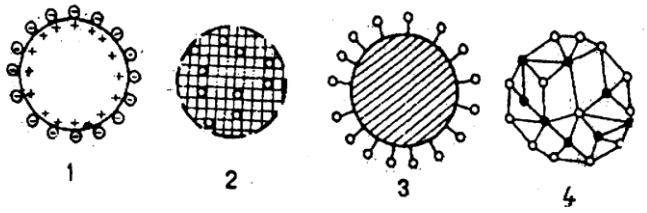
❖ *COVALENT BONDING*

Some fuctional groups, which are not essential for tha catalytic activity of an enzyme, can be covalently bonded to the support matrix (transducer or membrane). This method uses nucleophilic groups for coupling, such as NH_2, CO_2H, OH, C_6H_4OH and SH, as well as imidazole. For instance, a carboxyl group on the support is reacted with a carbodiimide. This then couples with an amine group on the biomaterial to form an amide bond between the support and the enzyme.

Such reactions need to be performed under mild conditions, i.e. low temperatures, low ionic strengths and pH levels in the physiological range.

The particular advantage of this method is that the enzyme will not be released during use. In order to protect the active site, the reaction is often carried out in the presence of the substrate.

Figure 2: Schematic diagram of immobilization methods used for biosensor construction. **1**. adsorption, **2.** gel entrapment, **3.** covalent binding to external surface, **4.** cross linking [3].



Overall, the lifetime of biosensor is greatly enhanced by proper immobilization. Typical lifetimes for the same biosensor, in which different methods of immobilization are used, differ from 1 day (for adsorption), via 1 week (membrane encapsulation), 3-4 weeks (physical entrapment), to 4-14 months (for covalent entrapment) [1]. Each of mentioned methods carries its own advantage for certain purpose. And in practice, there is also common use of their combination.

2.3.2. SOL-GEL IMMOBILIZATION

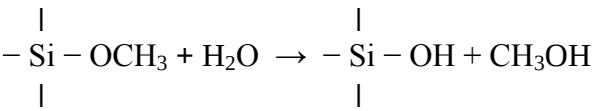
This kind of immobilization is one of the ways how to attach the enzyme into the electrode surface. In general definition, the *sol gel* technique starts with a colloidal suspension that can be gelled to form a solid. The resulting material is a porous, an optically transparent glass-like material although produced at low temperatures. Sol-gel glass has been increasingly used as a solid matrix for entrapment of chemical and biochemical agents (e.g. organic dyes, proteins, enzymes, microbial cells) in sensor development and other applications.

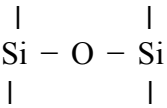
A good immobilization method should meet the following requirements:

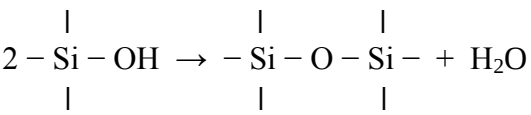
- be simple and fast;
- be nonspecific, i.e., the method can be used for the immobilization of various sensing agents;
- produce immobilized reagents that are stable and do not leach from the substrate;

- produce immobilized reagents that retain their chemical and biochemical activities.

In the sol-gel process, organometallic compounds (such as silicon alkoxides, e.g., $Si(OCH_3)_4$, tetramethylorthosilicate) are produced by the hydrolysis and polycondensation at low temperature. Silica glass prepared by the sol-gel method is a porous matrix that contains interconnected 'bottle-neck-like' pores (or so-called cavities and cages) formed by the resulting three-dimensional SiO_2 network. The pores are usually in the size range of 1.5 to 10 nm, depending on the composition of the precursor and conditions for preparation. It is found that the large sensing agents can be securely trapped but small analytes can diffuse readily into and out of the pores of sol-gel matrix. The sol-gel process can be divided into following steps: mixing (to form a solution), gelation, aging, and drying. In a typical procedure to prepare a silica glass by the sol-gel technique, one starts with an appropriate *alkoxide precursor*, e.g. $Si(OCH_3)_4$, which is mixed with water, an acidic catalyst such as HCl, and a co-solvent such as methanol, to form a solution (the sol) by stirring or sonication. Hydrolysis results in the partial substitution of the alkoxy terminals by hydroxyl groups - *silanol groups* (Si-OH).



The silanol groups react further to form *siloxane polymers* () in the condensation reaction.



Linkage of silanol with siloxane occurs as a polycondensation reaction and eventually leads to the formation of a SiO_2 network (with silanol groups on the surface). The resulting amorphous gel contains water and methanol.

Aging of a gel involves maintaining the gel immersed in liquid for a period of time, from hours to days. During aging, polycondensation continues and the strength of the gel thereby increases.

Finally, in the drying process, the solvents (water and methanol) are removed from the interconnected pore network.

A sensing agent is added to the mixture at sometime during the formation of the sol or gel. The sol-gel solution containing a sensing agent can be cast as monoliths, or it can be coated onto a silica or glass slide or an optical fiber to form a sensing element.

In the sol-gel glass, the reagents are entrapped inside the pores of the matrix where they may move freely inside the pores or they may have some interactions with silanol groups on the inner surface of the pores. The analytes to be determined, usually smaller, can diffuse into the pores and react with the sensing agents. On the other hand, the sol-gel glass has a large porosity (about 30%) and a very large specific surface area (>300 cm^2 /g). Therefore, a substantial fraction of the entrapped reagents may be exposed to a neighboring phase and intrapore volume and they can react with the analytes therein.

In the comparison with adsorption, covalent binding, and encapsulation methods using organic polymeric matrices, sol-gel glass as a solid matrix for chemical and biochemical sensing has folowing **advantages**:

1. compatibility with many inorganic and organic reagents;
2. chemical, thermal, photochemical and mechanical stability;
3. sol-gel is optically transparent (down to 250 nm), thus suitable for spectrophotometric and spectrofluorimetric measurements;
4. low temperature and mild chemical conditions for preparation of sol-gel, thus friendly conditions for proteins and enzymes;
5. stabilization effect of the solid matrix for some reagents entrapped in sol-gel glass, their life-time is longer than in solution;
6. possibility of various configurations of sensing elements for different applications; the sol-gel can be cast as monoliths, coated as thin films on slides and fibers, or ground into powder.

On the other hand, we can find some **disadvantages** of the sol-gel glass method:

1. a slow response of sol-gel sensors in aqueous media (in order of minutes). The response is limited by the diffusion process which is dependent on sizes of pores, thickness of the film, size and concentration af analyte, etc.;
2. leaching of reagents (particularly small molecules) may occur, although it is greatly reduced as compared to leaching problems of adsorption methods;
3. sol-gel glass prepared from tetrafunctional precursors will continue to condense long after gelation, this will lead to a decrease in the pore sizes with time and

affect the ability of analytes to diffuse into the sol-gel matrix, thus the response time and sensitivity of the sensor;

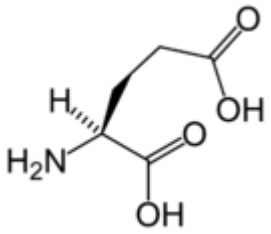
4. entrapment in sol-gel glass may slightly change spectroscopic and chemical properties and biological activities of the reagents, due to reduced degree of freedom in the pores, interactions with the inner surface of the pores or denaturation by the alcohols produced during the polycondensation step.

Sol-gel method has been a well developed technique in material sciences. Sol-gel glass has found its application among pH sensors, sensors for ionic species, gas and glucose, as a solid matrix for organic dyes, proteins and enzymes, yeast cells, etc. And other forms of applications are still in development. Furthermore, since sol-gel glasses are stable, clean and nontoxic, the fiber-optic sensors based on sol-gel glasses can be used in harsh enviroments, in food industries and in medical diagnoses [4].

2.4 GLUTAMIC ACID

Glutamic acid in its free form and monosodium glutamate (MSG) are used to enhance flavour in foods prepared at home, in restaurants, and by the food processing industry. Glutamate occurs in two optical isomeric forms; the L-form is the one that possesses the ability to enhance flavour [5].

Figure 3: Chemical structure of glutamic acid.



In 1907, Japanese researcher Kikunae Ikeda of the Tokyo Imperial University separated crystals of glutamic acid from the kombu broth. These crystals, when tasted, reproduced the ineffable but undeniable flavour he detected in many foods, most especially in seaweed. Professor Ikeda termed this flavour *umami* and along to his suggestion, *umami* was included in five main tastes (beside sweet, salty, bitter and sour). (The word *umami* in Japanese means “savory” or “meaty”.) He then patented a method of mass-producing a crystalline form of glutamic acid, MSG, and in 1909 the Ajinomoto company was established in Japan, which started producing of the first artificial flavour-enhancer ever.Modern commercial MSG is produced by fermentation of starch, sugar beets, sugar cane, or molasses [6].

Glutamate is a major component in all proteins, and occurs as a free acid in a variety of vegetables (such as tomatoes, legumes), nuts, mushrooms, meats and sea products. The range varies between 6.7 and 658 mg/100 g in fresh food to 0.05 and 6830 mg/ 100 g in processed foods (e.g., soy sauces, canned vegetables, soups, processed meats, instant meals and spices, certain sharp cheeses, etc.).

Glutamate also plays an essential role in many metabolic processes. Almost two kilograms of naturally occurring glutamate are found in muscles, in the brain, in kidneys, in the liver and in other organs and tissues. Studies have shown that the body

uses glutamate as a nerve impulse transmitter to the brain. On the other hand, injections of glutamate in laboratory animals have resulted in nerve cell damage.

The use of glutamic acid and MSG in the USA has become controversial in the past 30 years. Some people who have consumed food containing MSG have experienced adverse effects such as stiffness, tension, and pain in the head, neck, and shoulders. Persons with severe, poorly controlled, asthma may suffer temporary worsening asthmatic symptoms after consuming MSG. Although the acceptable daily intake of MSG is quoted as no more than 10.5 g, it was found that even a small dose of 2 g can induce asthma in some patients, and that there are large variations in oral threshold doses among individuals. In addition, some studies demonstrate a connection of MSG consuming with the appearance of neurological manifestations, such as Parkinson´s and Alzheimer´s disease.

The complaints and reports filed by the public regarding the use of MSG have engaged the USA Food and Drug Administration (FDA) in a review and analysis of collected scientific information. In 1994, FDA received a citizen´s petition requesting that the amount of free glutamate or MSG should be stated on the label of manufactured or processed food [5].

Up to the present day FDA has not answered to this petition, there is only duty to imprint the presence of glutamate in food products by a symbol ”E 621“ (in EU) or ”MSG“ (in USA).

However, if such quantitative information is required in future, the importance of the L-glutamic acid determination in samples will increase greatly.

2.4.1 DETERMINATION OF L-GLUTAMATE

Numerous analytical methods for determination of L-glutamate have been developed using different detection techniques and assay formats, e.g., spectrophotometry, fluorescence, chromatography, enzyme based electrode, and flow injection analysis (FIA) [7].

Enzymes are known for their profitable properties such as high selectivity and sensitivity. Therefore several methods using an enzyme for the determination of L-glutamate have been developed using various systems and detection techniques.

Three enzymes are used for the detection of L-glutamate: L-glutamate dehydrogenase (GDH), L-glutamate oxidase (GluOx) and L-glutamate decarboxylase (GDC). The following schemes show the process of the reactions.

$$\text{L-glutamate} + \text{NAD(P)}^+ + \text{H}_2\text{O} \xrightarrow{\text{GDH}} \text{2-oxoglutarate} + \text{NAD(P)H} + \text{H}^+ + \text{NH}_4^+$$

$$\text{L-glutamate} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GluOx/ FAD}} \text{2-oxoglutarate} + \text{NH}_3 + \text{H}_2\text{O}_2$$

$$\text{L-glutamate} \xrightarrow{\text{GDC}} \gamma\text{-aminobutyric acid} + \text{CO}_2$$

GDH requires for its activity the presence of NAD^+/NADH or $NADP^+$/NADPH cofactor. The reaction of GluOx can be facilitated by FAD.

L-glutamate can be at the end detected and determinated by several methods which are based on presence of reaction products. For instance, free ammonia, hydrogen or carbon dioxide can be measured by potentiommetric techniques. Hydrogen peroxide can be detected either directly or by the chromogenic peroxidase reaction or by an amperometric method. One could also measure decreasing oxygen at left side of the reaction.

In this report the use of L-glutamate dehydrogenase was selected in an atempt to develop a biosensor that in its final design would require only pH adjustment of sample to be assayed. For this purpouse, both the enzyme and cofactor should be entraped in the membrane of the sensor device. Regeneration of $NADP^+$ is ensured by reaction with polymethylene green.

2.4.2 L-GLUTAMATE DEHYDROGENASE AND NICOTINAMIDE ($NAD(P)^+$/NAD(P)H) COENZYME

Glutamate dehydrogenase (GDH; EC 1.4.1.3) is a mitochondrial matrix enzyme, expressed at high levels in liver, brain, pancreas and kidneys, but not in muscle. As

mentioned above, it catalyses the reversible oxidative deamination of glutamate to 2-oxoglutarate plus free ammonia using either NAD^+ or $NADP^+$ as a cofactor [8].

The enzyme belongs to so-called *pyridine-linked dehydrogenases.* This class of enzymes catalyzes the general reaction:

$$\text{substrate}_{\text{RED}} + \text{NAD(P)}^+ \leftrightarrow \text{substrate}_{\text{OX}} + \text{NAD(P)H} + \text{H}^+$$

The reaction involves reversible transfer of two electrons and a proton and can formally be considered to be a hydride transfer. Most of the pyridine-linked dehydrogenases are specific for either NAD^+ or $NADP^+$, but a few of them, including glutamate dehydrogenase, can react with both coenzymes [9].

When the enzyme is operating in the reaction and $NAD(P)^+$ or NAD(P)H is transformed to its reduced or oxidised form, respectively, we need to regenerate the coenzyme in order it could work in all process again. This problem of coenzyme recycling can be solved in several ways. At first, coenzyme regenaration can be accomplished by use of added *chemical oxidising or reducing agents*.

The second way of solution is *coupling the enzymatic reaction* producing $NAD(P)^+$ with another enzymatic reaction which consumes NAD(P)H. (For example galactose dehydrogenase, alanine dehydrogenase, and dextran bound NAD^+ were immobilised in a bioreactor, which produced alanine and galactonate from pyruvate and galactose.

An alternative way of regeneration is the *electrochemical oxidation*. The formal redox potential of the $NAD(P)^+$/NAD(P)H couple is –0.56 V vs. SCE (at pH 7.0) but the first intents of direct NADH oxidation on solid carbon electrodes demonstrated that very high overpotential must be applied (+0.5 – 0.7 V vs. SCE). The speculated mechanism of the oxidation reaction on bare electrodes is:

$$\text{NAD(P)H} - e^- \leftrightarrow \text{NAD(P)H}^{\cdot +}$$
$$\text{NAD(P)H}^{\cdot +} + \text{B} \leftrightarrow \text{NAD(P)}^{\cdot} + \text{BH}^+$$
$$\text{NAD(P)}^{\cdot} \leftrightarrow \text{NAD(P)} + e^-$$

where B is a base and $NAD(P)H^{\cdot +}$ and $NAD(P)^{\cdot}$ are the intermediate radicals.

It is very possible that a disproportionation reaction also takes place:

$$NAD(P)^{\cdot} + NAD(P)H^{\cdot +} \leftrightarrow NAD(P)^{+} + NAD(P)H$$

Considering the oxidation of NAD(P)H by air and at high potentials can form also dimmer which does not allow reusing, one can see that lowering the applied potential for NAD(P)H oxidation is very desirable. For managing this aim we can use few methods. One of the options is to employ enzymatic recycling of reduced cofactors by diaphorases or NAD(P)H oxidases according to the following sequence of reactions:

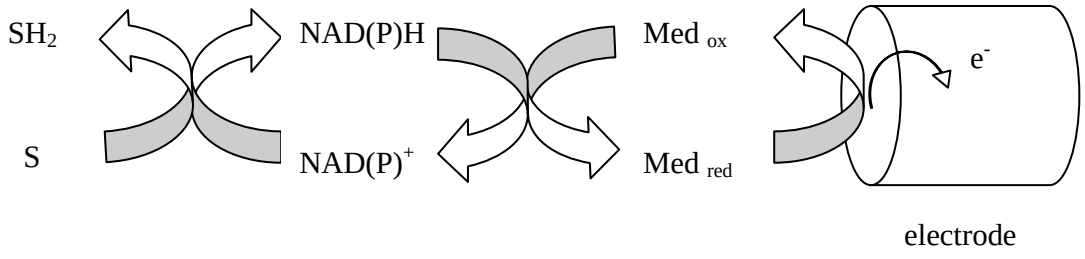
$$NAD(P)H + Diaphorase_{ox} \leftrightarrow NAD(P)^{+} + Diaphorase_{red}$$
$$Diaphorase_{red} + Mediator_{ox} \leftrightarrow Diaphorase_{ox} + Mediator_{red}$$

and, finally, the reoxidation of a mediator on the electrode surface:

$$Mediator_{red} \rightarrow Mediator_{ox}$$

Another possible solution to the problem of high overpotential is to introduce into the system catalysts for the oxidation of NAD(P)H. They increase the rate of processes by introducing new mechanisms with lower activation energies mediating the transfer of electrons from NAD(P)H to the electrode surface and are referred as mediators which operate according to Fig. 4. The function of mediator is to serve as a catalyst for NAD(P)H oxidation. NAD(P)H is oxidised by the mediator, and the mediator is oxidised in its turn on the electrode surface at low applied potential [10].

Figure 4: (S is for substrate, Med for mediator)



2.4.3 MEDIATORS AND METHYLENE GREEN

As explained above, the mediator is a catalyst which is used for oxidation of NAD(P)H at low applied potential. For the role of mediator we can employ many chemical substances. They can be classified into ‘natural’ and ‘artificial’ electron mediators. The former type includes molecules as the cytochromes, ubiquinone, flavoproteins and ferridoxins, while artificial mediators include many dyestuffs, such as methylene blue, ferrocene, phthalocyanines and viologens [1].

For our experiments we chose an artificial water-soluble dye – methylene green (MG), which chemical structure is very similar to methylene blue. In the case of MG, the position 4 of a molecular skelet is substituted by the nitro functional group. Our solution of MG was prepared with the zinc chloride double salt (see Fig. 5).

Figure 5: Methylene green, zinc chloride double salt [11].

(7-dimethylamino-4-nitro-phenothiazin-3-ylidene)-dimethyl-azanium

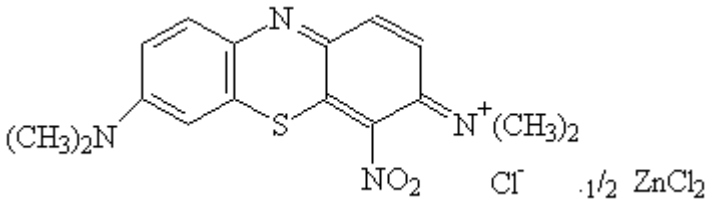
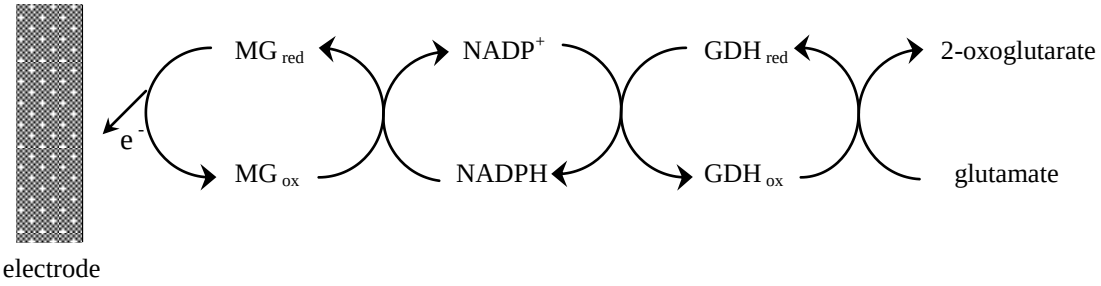


Figure 6: The schematic mechanism of our reaction.



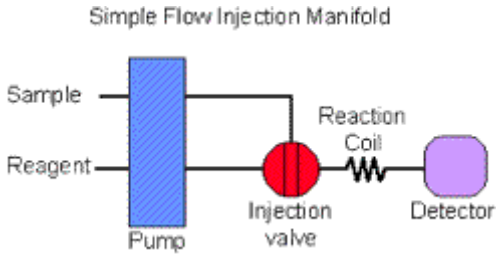
2.5 GENERAL DESCRIPTION OF FLOW INJECTION ANALYSIS

As flow injection analysis (FIA) was settled to provide automated measurements with the biosensor in its different development steps. Flow-injection analysis provides the ground concepts for more recent continuous-flow techniques such as sequential-injection analysis (SIA), multicommutation binnary sampling analysis or lab-on-valve analysis. It is however more easy to implement because it does not require computer control of its different constituting devices to get fast and accurate analytical results.

A typical industrial definition describes FIA as: *"a simple and versatile analytical technology for automating wet chemical analysis, based on the physical and chemical manipulation of a dispersed sample zone formed from the injection of the sample into a flowing carrier stream and detection downstream."*

The following diagram (Fig. 7) can be used to describe the basic components and principles of FIA.

Figure 7: Diagram describing the basic components of FIA [12].



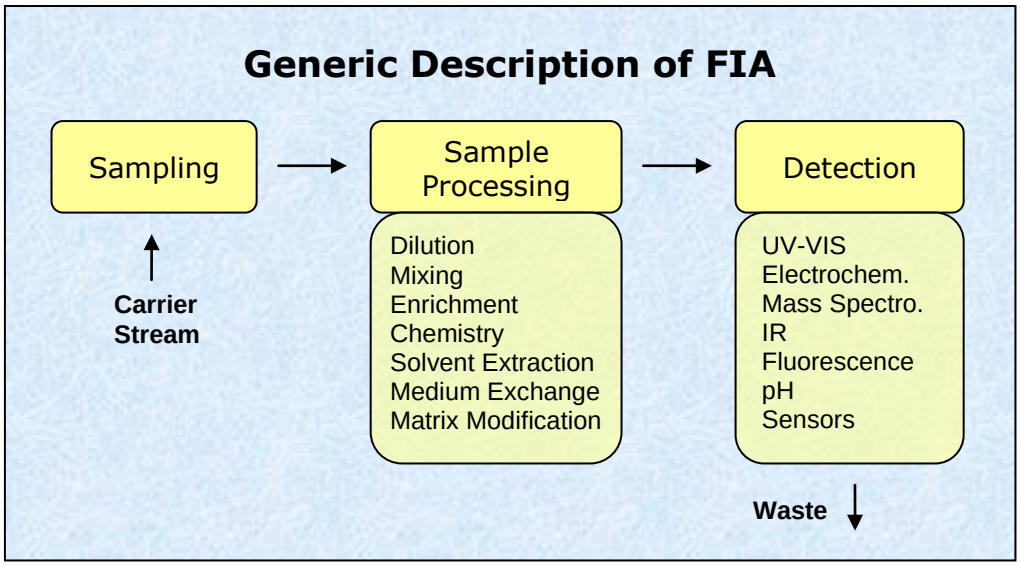
A typical FIA manifold is comprised of a pump, injection valve, detector, and tubing manifold. The pump is used to propel continuously one or more streams through the detector via narrow bore (0.5 - 0.8 mm i.d.) tubing. These streams may be reagents, solvents, or some other medium such as a buffer. The injection valve is used to periodically introduce a small volume (generally <100 µl) of sample into the carrier stream. As this sample is carried to the detector, the fluid dynamics of flow through narrow-bore tubing mixes sample and reagent, leading to chemical reaction to form a detectable species. Regarding that the transport of the sample bolus through the reaction coil occur under laminar flow conditions, mixing with the reagents present in the carrier are produced mainly by diffusion gradients at the front and rear interfaces of the sample. The reaction products are then sensed by the detector as a transient peak. The

height and area of the peak are proportional to concentration, and are used to quantify the concentration of the compound being determined by comparison to samples of known concentration (calibration curve).

It is possible both to carry out the above process in a manual system with detector output connected to a chart recorder, or resorting to a computer with appropriate data acquisition and manipulation capabilities.

In a similar fashion to manual conducted procedures (batch procedures) the FIA process can be grouped into three stages as indicated in the schematic diagram below (Fig. 8).

Figure 8: Diagram describing process of FIA.



First is *sampling*, where a precise and accurate volume of sample to be assayed is measured out and intercalated into the flowing carrier stream (the name Flow Injection Analysis was preserved because in the first papers samples were inserted using seringes). This step is generally performed with a sample injection valve being the volume of sample defined by the physical dimensions of the loop.

The second stage is what we call *sample processing*. The purpose of this step is to transform the analyte into a species that can be measured by the detector or isolate it from the interfering sample matrix, and manipulate its concentration into a range that is compatible with the detector, using one or more of the indicated processes.

The third stage is *detection* where the analyte, or a derivative of it, generates a signal peak that is used to quantify the compound being determined. As indicated, a large

variety of detectors can be used in FIA. Detection is most frequently photometric (UV/VIS). Besides, different luminescence techniques are gaining popularity once due to the transient nature of luminescence phenomena procedures reproducibility are required to enable quality analytical information. Electrochemical techniques such as amperometry, and potentiometry, have gained new importance by coupling them to flow-based sample handling techniques such as FIA and SIA.

The first and last stages are, largely, conventional technology. It is the second stage, sample processing, that is the heart of FIA. A number of the most common analytical sample processing functions that can be performed by FIA are depicted in diagram above. For example, FIA can dilute by factors up to tens of thousands, and can enrich by several hundred. It can perform chemistry on an analyte to generate a detectable species. It can transfer an analyte from one medium to another, for example from a gas sample to a FIA carrier, and vice versa. It can do solvent extraction, and matrix modification or matrix elimination [12].

FIA was defined by Růžička and Hansen in 1975, in a time where computer technology was mostly absent of chemical laboratories. Růžička with Marshall then developed another method inferred from FIA, well suited for computer control which was SIA in 1990. The chance to simplify procedures optimization or extent the application of the system to different determinations only by changing injected volumes and carrier flow rates software through software instructions were the most important advantages.

3 EXPERIMENTAL PART

3.1 LIST OF CHEMICALS

Glutamic dehydrogenase from bovine liver (Sigma)

β-Nicotinamide adenine dinucleotide phosphate sodium salt (β-$NADP^+$) (Sigma)

β-Nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), tetrasodium salt (Sigma)

L-Glutamic acid (Aldrich)

Potassium chloride (Sigma-Aldrich)

Sodium dihydrogen phosphate monohydrate (Merck)

di-Sodium hydrogen phosphate dihydrate (Fluka)

Hydrochloric acid 37% PA (Pronalab)

Methylene green zinc chloride double salt (Fluka)

(3-Aminopropyl)trimethoxysilane (Fluka)

2-(3,4-Epoxycyclohexyl)ethyltrimethoxysilane (Fluka)

Polyethylene glycol 6000 (Fluka)

Tetraethyl orthosilicate (TEOS) (Fluka)

Ethanol (Riedel-da-Han)

Glycerol (Riedel-da-Han)

Araldit® M for electron microscopy epoxide-based embedding agent (Merck) (used like ”resin“)

REN HY 956, curing agent for epoxy resins (Vantico) (used like ”hardener“)

Graphite fine powder extra pure (Merck)

3.2 THE PREPARATION OF SOLUTIONS

For the preparation of all solutions, reagents of p.a. quality or similar as well as deionised water (Millipore Milli-Q system) were used. Analytical grade chemicals were used without further purification.

Solutions of $NADP^+$, NADPH and enzyme were prepared freshly and stocked in dark place at 4°C. Buffer solutions were prepared as stock solutions and stored at a room

temperature. Stock solutions of methylene green and L-glutamate were kept at 4 °C without light.

BUFFERS COMPOSITION

a) Phosphate-Buffered Saline (PBS) buffer, 0.1 M:

- pH 5.6: 230 ml of acid component (NaH_2PO_4, 0.2 M)
  20 ml of basic component (Na_2HPO_4, 0.2 M)
  3.728 g KCl (final concentration in solution was 100 mM)
  made up to 500 ml with pure water

- pH 7.4: 202.50 ml of acid component (NaH_2PO_4, 0.2 M)
  47.50 ml of basic component (Na_2HPO_4, 0.2 M)
  3.728 g KCl (final concentration in solution was 100 mM)
  made up to 500 ml with pure water

- pH 8.0: 13.25 ml of acid component (NaH_2PO_4, 0.2 M)
  236.75 ml of basic component (Na_2HPO_4, 0.2 M)
  3.728 g KCl (final concentration in solution was 100 mM)
  made up to 500 ml with pure water

ENZYME SOLUTION

Glutamic dehydrogenase was rigorously weighed and dissolved in PBS buffer (0.1 M, pH 7.4) to gain the various concentrations. The activity of enzyme is 16.8 units/mg. The prepared enzyme solution was then stored at 4 °C.

3.3 APPARATUS

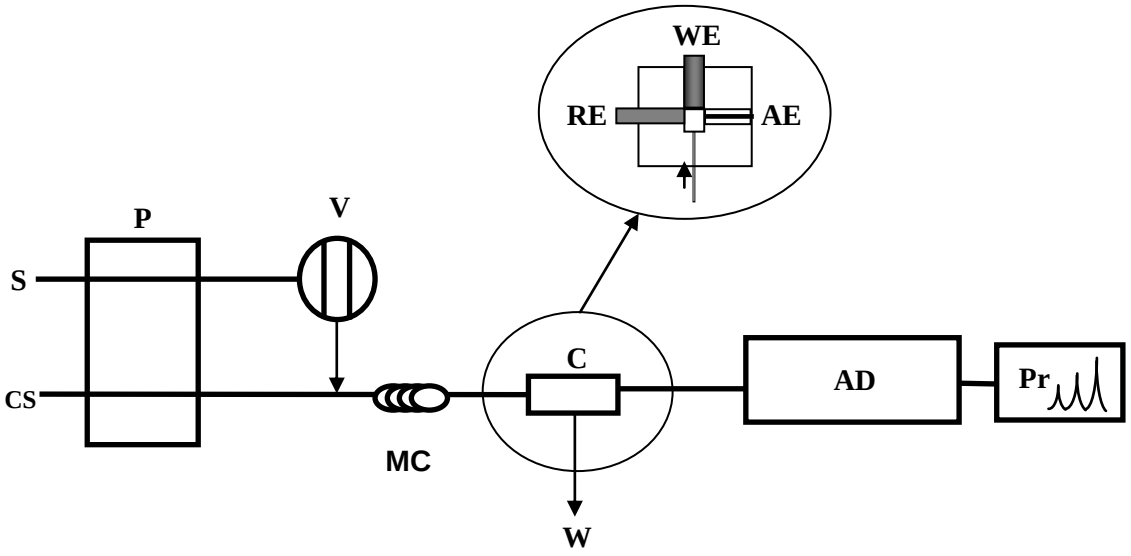
For spectrophotometric studies the UV/VIS Spectrometer (Perkin Elmer, Lambda 45) was used, controlled by UV Winlab 2.85.04 software.

The electrochemical measurements were performed with a potentiostat (PGSTAT 10 Ecochemie/ Autolab) controlled by GPES 4.8 software. For general opening batch studies a 663 VA Stand Metrohm was used, consisting of a working sol-gel sensor, a reference Ag/AgCl (KCl sat.) electrode and an auxiliary glassy carbon electrode.

The evaluation of sensor performance under flow conditions were performed with a simple flow-injection system (Fig. 9), using a Gilson Minipuls 2 peristaltic pump (P) (Viliers-le-Bell, France), a Rehodyne 7050 injection valve (V) and a flow cell (wall-jet 656 VA Metrohm Stand electrochemical detector) - (C) with three electrodes – graphite electrode electropolymerized with MG (working electrode, WE) and Ag/AgCl (KCl sat.) and Au, as a reference (RE) and auxiliary (AE) electrodes, respectively. A mixing coil (MC) made of PTFE tubing (0.8 mm inner diameter, 25 cm length) connected the injection valve to the detection flow cell.

With FIA system we made several studies, results of two of them are presented in this report.

Figure 9: Schematic diagram of the FIA manifold. S-sample; CS-carrier solution; P-peristaltic pump; V-injection valve; MC-mixing coil; C-flow cell; RE-reference electrode; WE-working electrode; AE-auxiliary electrode; AD-amperometric detector; Pr-printer, W-waste line.



4 RESULTS AND DISCUSSION

4.1 SPECTROPHOTOMETRIC STUDY

Initially a prospective study was conducted in order to evaluate simultaneously the effect of the catalytic oxidation of L-glutamate on both the $NADP^+$/NADPH pair and MG levels. It would be indicative not only of the possibility of using MG to regenerate NADPH, but also of its efectiveness.

For this study the following solutions were prepared.

Reagents (all components are disolved in PBS buffer 0.1 M, pH 7.4)**:**

Composition in the 1st cuvette

100 μl	Methylene green ($1x10^{-4}$ M)
2900 μl	PBS buffer 0.1 M, pH 7.4

Composition in the 2nd cuvette

100 μl	Methylene green ($1x10^{-4}$ M)
50 μl	L-glutamate dehydrogenase (2.63 mg/ 0.5 ml) = 88.37 units/ ml
25 μl	$NADP^+$ (1.323 mM)
2825 μl	PBS buffer 0.1 M, pH 7.4

Composition in the 3rd cuvette

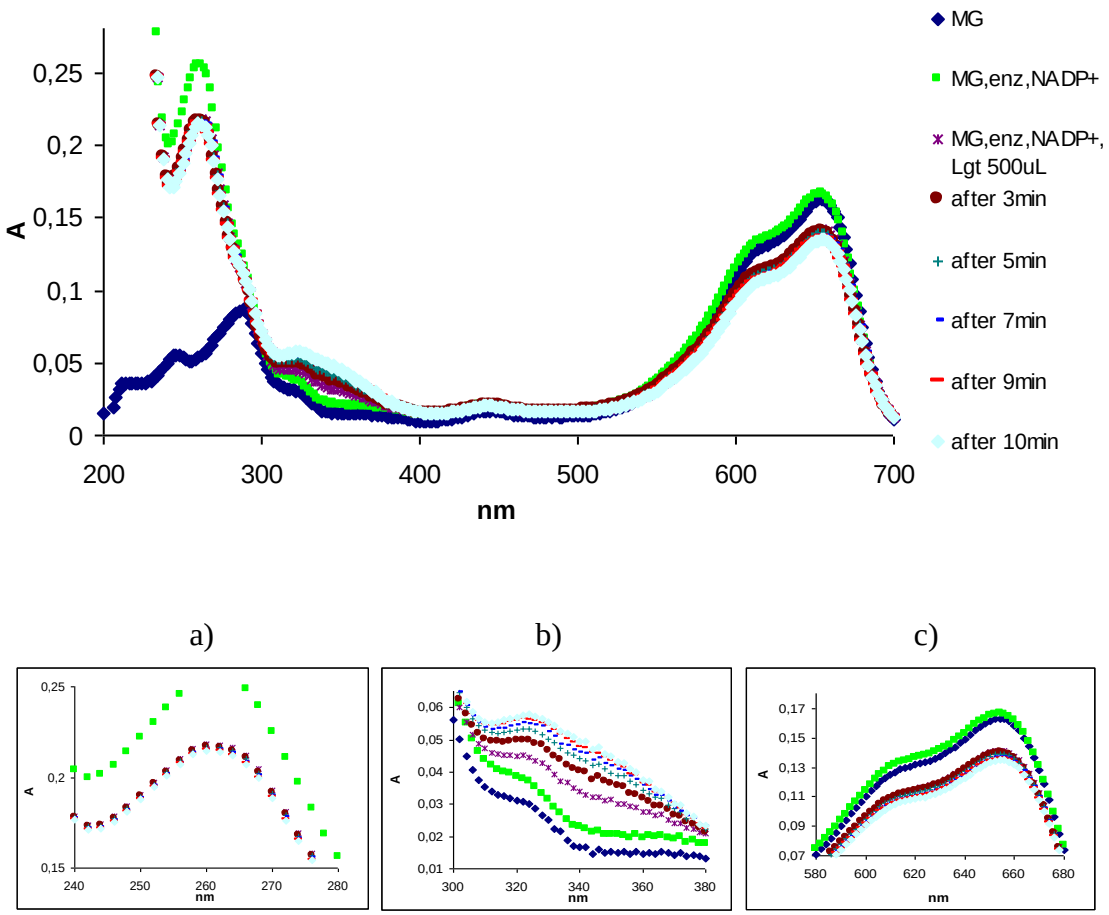
100 μl	Methylene green ($1x10^{-4}$ M)
50 μl	L-glutamate dehydrogenase (2.63 mg/ 0.5 ml) = 88.37 units/ ml
25 μl	$NADP^+$ (1.323 mM)
500 μl	**L-glutamic acid (10 mM)**
2825 μl	PBS buffer 0.1 M, pH 7.4

Firstly, two cuvettes were filled with PBS buffer 0.1 M, pH 7.4 as a blank, in order to set the zero baseline of the dual beam spectrophotometer. Then the cuvette in sample compartment position was changed by each one of the previous described cuvettes. Absorbance of these three mixtures was measured depending of increasing wavelength against PBS buffer 0.1 M, pH 7.4 as a blank (Fig. 10).

The study showed the typical absorption curve of methylene green (MG, the mediator of NADPH regeneration). In the presence of MG the other participants of the reaction behaved as shown in the picture. It is known that $NADP^+$ absorbs at 260 nm and

NADPH at 340 nm. Looking at the graph we can see that the reaction was running and was not negatively effected by the presence of MG. The working ability of enzyme was not affected, either. The amount of $NADP^+$ was lightly decreasing (inset a, Fig. 10), oppositely, NADPH was increasing accordingly (inset b, Fig. 10). The MG mediator was working, as its curve was decreasing in time (inset c, Fig. 10), but its work was not efficient enough to oxidate all NADPH produced in the reaction. The reaction medium would probably need to level up the amount of MG in reaction and possibly to adjust the buffer concentration and pH.

Figure 10: Absorption behaviour of a reaction mixture.



Based on the indications provided by these simple spectrophotometric trials it was decided to proceed by implementing and optimizing the proposed scheme now with electrochemical transduction.

4.2 ELECTROCHEMICAL MEASUREMENTS

4.2.1 CONSTRUCTION OF A LOW RESISTANCE BIOSENSOR BODY

For our experiments we needed to construct two types of support biosensor body. One was intended for *cyclovoltammetric measurements* in the glass cell, thus it was not limited in size as much as the other, which was supposed to fit to the exact sized detection flow cell of *FIA system*.

For the former one we used the precision pipette tips of 1ml volume, which were cut on the top to gain a diameter of 3-4 mm. The tip was then filled with a mixture of graphite powder, resin and hardener (1:0.2:1.75) to the high of about 15 mm together with a conducting wire fixed very thoroughly. The graphite filling was then pressed properly to prevent the presence of cavities inside (cavities inside of graphite fill could cause increase in electrode resistance). Graphite electrodes prepared this way were left to dry for 24 hours at 50 °C. Afterwards, we covered the surface of graphite filling (the part which is not in contact with electrolyte) with a mixture of resin and hardener (1:0.2) for protecting and proper fixing. Then we left it to dry at room temperature. Before use, the surface of electrodes was manually polished with sand paper, washed with deionised water and cleaned by ultrasound for 5 min. The resistance of good-working electrodes should be as lowest as possible (desirebly bellow 100 Ω) in order to enable measurement of low current intensities.
The later type of electrode was constructed in the same way using a Teflon body instead of the pipette tips.

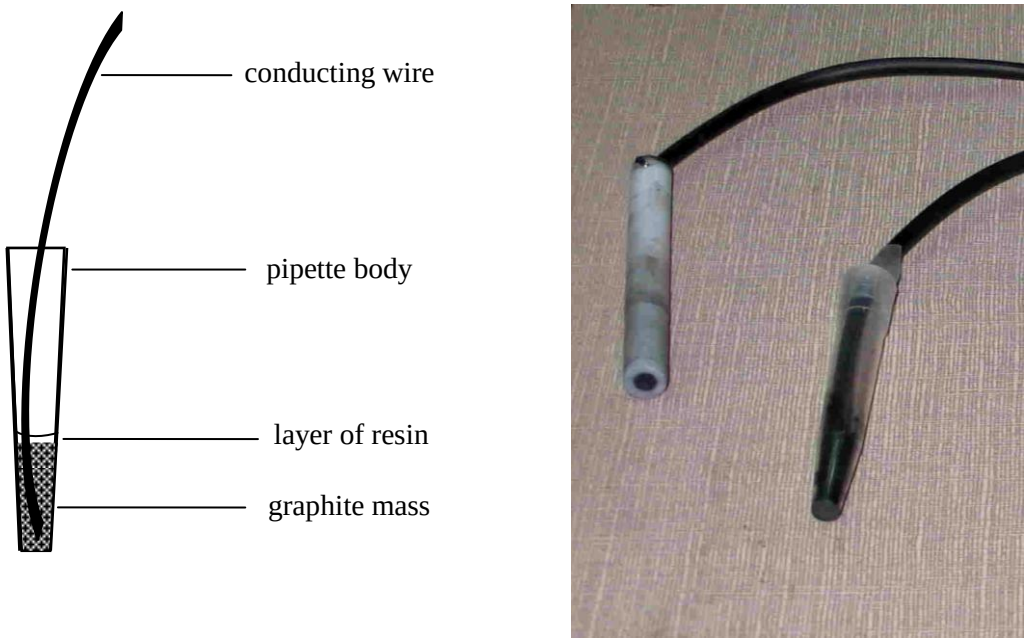
In this process we followed the previous work [13] with some modifications (such as higher amount of graphite powder).
The composition of graphite mixture used was:

1 g	resin (i.e. Araldit® M (Merck))
0.2 g	hardener (i.e. REN HY 956 (Vantico))
1.75 g	graphite powder (i.e. Graphite fine powder extra pure (Merck))

At first the resin and the hardener were mixed together, then the graphite powder was added and mixed till a homogenous mixture was obtained.

After electrode preparation and after each measuring we washed up used electrode 5 min by manual polishing on a microcloth pad (Buehler) with Alpha Micropolish® II deagglomerated Alumina suspension of 0.3 micron (Buehler). Afterwards, we polished it again on another microcloth pad only with pure water. The cleaning was ended by ultrasonication for 5 min in water.

Figure 11: Scheme and photo of constructed graphite electrodes.



4.2.2 MODIFICATION OF THE BIOSENSOR BODY BY ELECTROPOLYMERIZATION OF METHYLENE GREEN

Graphite electrodes were modified in order to be covered with a poly(methylene green) thin film by means of methylene green electrochemical polymerisation, using a cyclic voltammograms based procedure. The set-up used a traditional three-electrode cell. The Ag/AgCl (KCl sat.) electrode was used as reference electrode and glassy carbon electrode as auxiliary electrode. The graphite electrode (its construction is described above) served as working electrode. Electrodes were carrying by a VA Stand Metrohm device, which cooperated with a potentiostat (PGSTAT 10 Ecochemie/ Autolab) controlled by GPES 4.8 software.

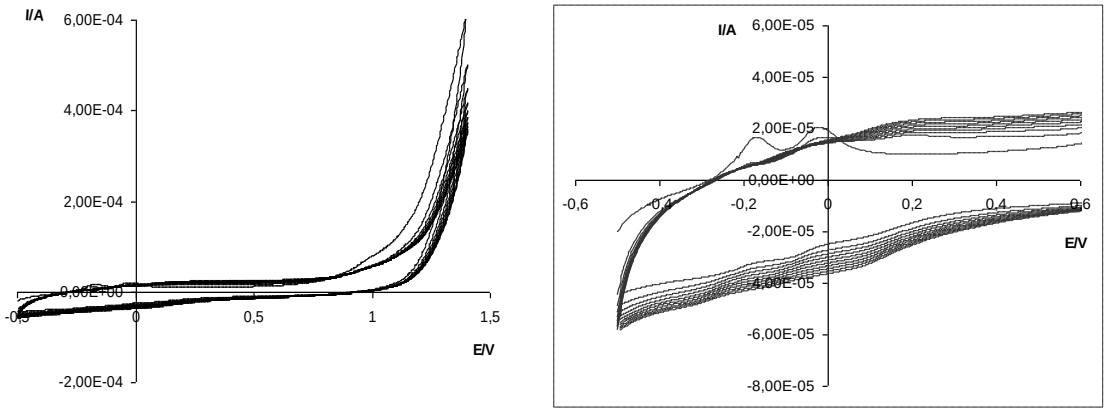
The process of electrode modification can be divided into three parts: *pretreatment, polymerization* and *stabilization* [14]. First of all, the cleaned graphite electrode was pretreated by continuous cyclic sweeps from –0.545 to +0.955 V (vs. Ag/AgCl, KCl sat.) at 50 mV/s in PBS buffer (pH 5.7) during 100 scans.

The self polymerization was held under cyclic sweeping from –0.5 to +1.4 V (vs. Ag/AgCl, KCl sat.) at 50 mV/s in PBS buffer (pH 7.4) containing 5.0×10^{-4} M methylene green during 50 scans.

After that, the modified electrode could either undergo stabilization cyclic sweeps from –0.5 to +0.5 V, at 50 and 10 mV/s in PBS buffer (pH 7.4) until a constant background was obtained and then used for further studies, or be used for creating a sol-gel glass membrane on its surface. In the later case, the stabilization process was performed as lately as the sol-gel layer was formed.

In comparison with the original article [14], we had to adapt some steps to our conditions. For example, we needed to use higher potential for MG polymerization (+1.4 V), the primary potential of +1.2 V was not sufficient, because either the reference electrode and the material of working electrode were different. According to the paper we were supposed to observe the first cycle of cyclic voltammogram showed two clear couples of redox peaks, which are produced by the redox reaction of the monomer of MG, but in the following cyclic voltammograms the two couples of peaks should disappeared, and at the same time, wide range peaks in more positive direction should obtain due to the polymerization. Thus, higher potentials were tried until achievement of a similar behaviour (Fig. 12).

Figure 12: Cyclic voltammograms of 5.0×10^{-4} M methylene green in pH 7.4 PBS buffer between – 0.5 and + 1.4 V (vs. Ag/AgCl, KCl sat.) at an electrode modified by adsorption. Scan rate 50 mV/s; scan cycle 10.



4.2.3 DEVELOPMENT OF SOL-GEL GLASS BIOSENSORS

The modified electrodes (preparation explained in previous chapter) were used for creating a sol-gel glass membrane on their surface. We assayed to test two types of sol-gel glass in which the glutamate dehydrogenase was then immobilised. Final results presented in this report were obtained with electrodes covered with the first type sol-gel only; the second type of sol-gel is still in the phase of testing.
The method how to incorporate $NADP^+$ cofactor is in development, as well.

The first type of sol-gel glass used was prepared using an optimum concentration of (3-aminopropyl)trimethoxysilane, 2-(3,4-epoxycyclohexyl)ethyltrimethoxysilane, polyethylene glycol 6000, double distilled water and HCl, according to previous work in which alkyl substituted monomers were resorted to produce less hydrophilic silica nets [15].

The composition of sol-gel:

(3-Aminopropyl)trimethoxysilane	70 µl
2-(3,4-Epoxycyclohexyl)ethyltrimethoxysilane	20 µl
Polyethylene glycol 6000 (2 mg/ml)	700 µl
Hydrochloric acid (0.1 M)	7 µl
Double distilled water	700 µl

The components of sol-gel mixture were mixed properly by vortex and also with help of ultrasonication for 25 min. The various volumes corresponding to 5 µl and 7 µl of the homogenized solution were placed on the surface of the graphite electrodes. Gelation took place at room temperature for 60 min. Afterwards, 5 µl or 7 µl of enzyme solution of various concentration was added to the layer of the sol-gel glass. Then an incubation period of 24 hours was held. After that the electrodes were washed with PBS buffer (0.1 M, pH 7.4) and stored in the same buffer at 4 °C when not in use.

The other type of sol-gel glass containing poly(methyl silicate) (PMS) and poly(glyceryl silicate) (PGS), which is still at the beginning of our testing, was prepared with following process [16].

The composition of sol-gel:

TEOS (0.48 mol)	20 ml
Ethanol	9.25 ml
Hydrochloric acid (0.25 M)	2.0 ml
Glycerol (0.38 mol)	5.2 ml

Tetraethyl orthosilicate (TEOS) was mixed with ethanol, and hydrochloric acid was added over 30 min with vigorous stirring; then the mixture was heated to 70 °C for 15 h. Rotary evaporation at 35 °C provided PMS of composition $SiO_{1.1\text{-}1.2}$ $(OMe)_{1.6\text{-}1.8}$ as a clear, viscous liquid. PGS was obtained by adding glycerol to the reaction mixture over 1 h, heating to 50 °C, and stirring for a further 40 h. Then we took 900 µl of this precursor solution and mixed properly (for 2 min) with 100 µl of enzyme solution (8 mg/ 5 ml). Drops of 5 µl and 10 µl of this mixture were then spread over the electrodes surface. We also tried to immerse electrodes into this solution directly.

Another experiment was to make a film of precursor solution (5 µl) only being covered with enzyme solution (5 and 10 µl) after 30 min. In all experiments, 24 hours incubation was kept. Washing with PBS buffer and storing at 4 °C was carried out in the same way as in the case above.

4.2.4 CYCLOVOLTAMMETRIC MEASUREMENTS

BEHAVIOUR OF MG MODIFIED ELECTRODE TOWARDS NADPH

Cyclovoltammetric studies of graphite electrode for the biosensor construction, modified by methylene green electropolymerization (similar results were obtained either with and without casting a sol-gel layer over the film), were then performed resorting to the same potentiostat. A solution of 35.90 mg NADPH in the 0.1 M PBS buffer was examined. At first NADPH powder was dissolved in 5.0 ml to gain concentration of 8.62 mM. And this solution was after 3 min of stirring measured. In the next step the solution was diluted with PBS buffer to have always lower concetration of NADPH and after stirring for 3 min the study was repeated in the same way. The solutions of concentration between 0.0999 and 8.620 mM were then tested.
The potential was scanned from – 0.5 V to + 0.5 V vs. Ag/AgCl (KCl sat.) at 10 mV/s.
By this way we made a summary of scans of various NADPH concentrations and after that a calibration curve was made.

We could observe very well change in current in the presence of NADPH cofactor. This change was visible at potential of + 0.2 V which proved that high potential for NADPH oxidation was decreased (from original + 0.7 V without help of the mediator) and by this way we could lower the possibility of interferences with reagents.
The peak intensity increase depended on NADPH concentration. From the calibration curve we could see that this dependence was proportional and a good linear relation up to 8.62 mM was obtained.

Figure 13: Cyclic voltammetric sweeps between – 0.5 and + 0.5 V (vs. Ag/AgCl, KCl sat.); scan rate of 10 mV /s; NADPH solution prepared in PBS buffer 0.1 M, pH = 7.4, KCl 100 mM.

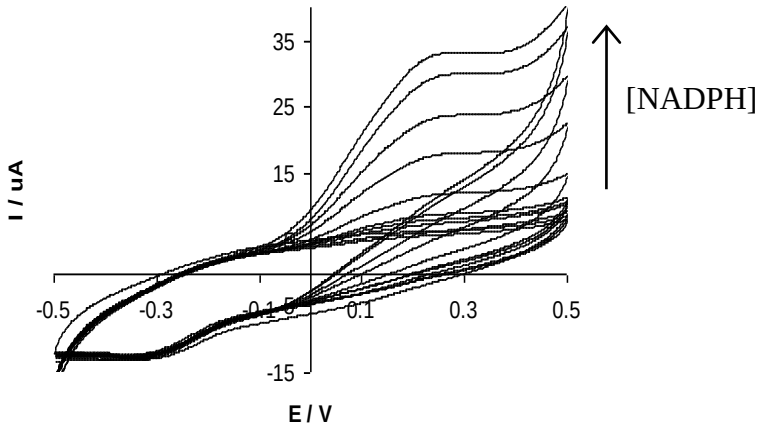


Figure 14: Calibration curve of NADPH measured by cyclic voltammetry (batch).

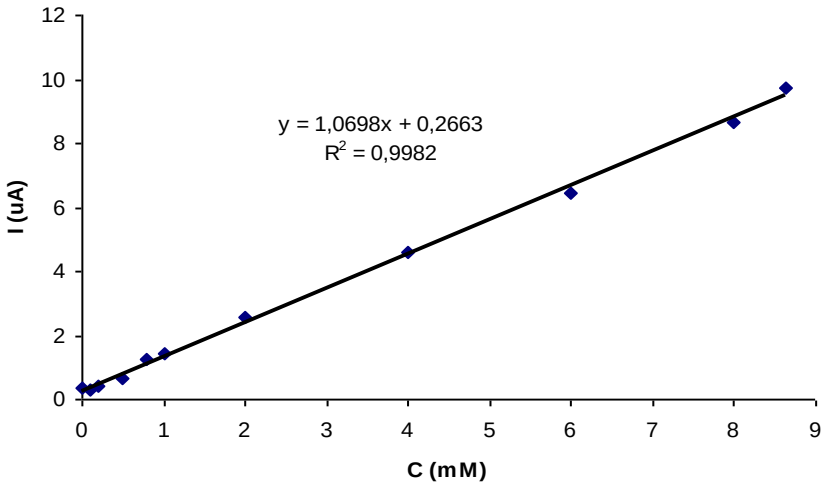


Table 3: Values of measured current representing the calibration curve (Fig. 14).

[NADPH]	Peak intensity	
c (mM)	I (A)	I (μA)
0.0000	3.34E-07	0.334
0.0999	2.83E-07	0.283
0.1999	4.14E-07	0.414
0.4997	6.50E-07	0.650
0.7996	1.23E-06	1.23
0.9995	1.42E-06	1.42
1.999	2.55E-06	2.55
3.998	4.59E-06	4.59
5.994	6.46E-06	6.46
7.996	8.65E-06	8.65
8.620	9.74E-06	9.74

4.2.5 AMPEROMETRIC MEASUREMENTS

In a similar way as above the performance of the surface modified electrode (now with the sol-gel glass layer) was assessed in flow conditions using amperometric transduction. In this mode, a fixed potential between the indicating and reference electrodes is settled and the current intensity produced by chemical processes at indicating electrode surface registered. The studies were carried out at + 0.2 V vs. Ag/AgCl electrode, KCl saturated. This potential corresponds with the potential of NADPH oxidation which was determined by the previous cyclovoltammetric study.

NADPH solutions were prepared in PBS buffer 0.1 M, pH 7.4 in concentrations between 2.696×10^{-3} mM and 10.74 mM.

Optimisation of the flow-injection system was initially performed in order to achieve the highest sensitivity. For this the reaction coil was fixed at 25 cm length and injection sample loop was increased by connecting PTFE tubing with 0.8 mm internal diameter with increasing lengths (≈ 5 μl/cm), until achievement of signals with intensity independent of the injection volume (> 200 μl). Then the carrier flow rate was increased until 1.4 ml/min. For higher flow-rates the sampling rate was higher but a reduction of the analytical signal was noticed.

The amperometric response proved that the electrode was treated (with MG film and sol-gel glass layer) in the right way and whole instrument was working well in FIA, as

well as in batch. The electrode was sensitive to NADPH (even to very low concentration) and cofactor oxidation was taking place at + 0.2 V as before.
The electrocatalytic current was proportional to the NADPH concentration and a good linear relation up to 10.74 mM was gained.

Following pictures (Fig. 15, 17) show the amperometric behaviour of NADPH solutions during two assays using two different ranges of concentration. From the measured values the calibration curves (Fig. 16, 18) were made consequently.

Figure 15: Applied potential of + 0.2 V (vs. Ag/AgCl, KCl sat.); NADPH solutions prepared in PBS buffer 0.1 M, pH 7.4 between **2.696 x 10^{-3} mM** and **0.8087 mM**; flow rate: 1.4 ml/min.

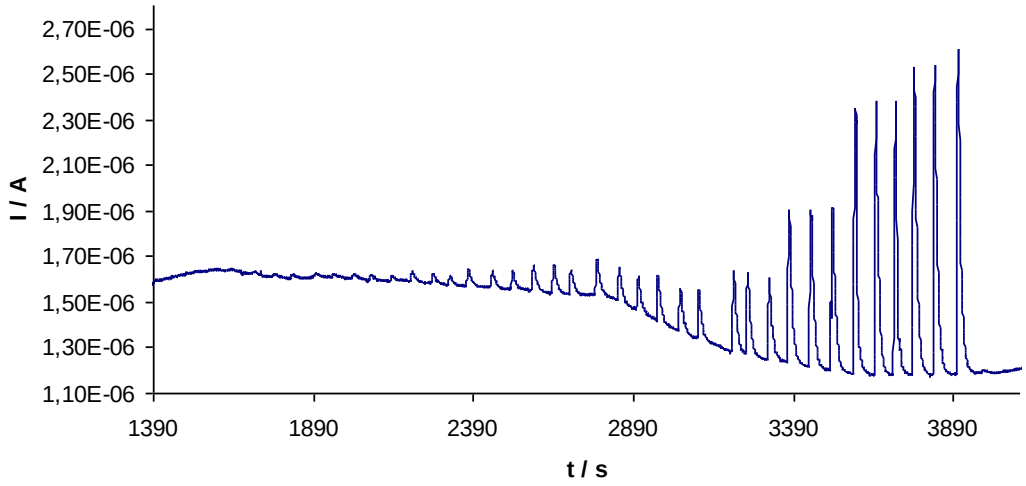


Figure 16: Calibration curve of NADPH measured by amperometry (FIA).

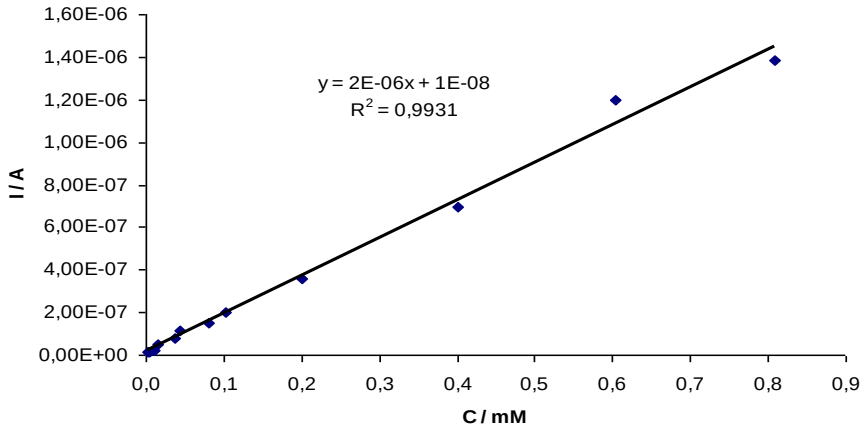


Table 4: Values of measured current representing the calibration curve (Fig. 16).

[NADPH]	Current media
C (mM)	I (A)
2.70E-03	1.21E-08
5.39E-03	1.47E-08
8.09E-03	1.98E-08
0.01078	2.23E-08
0.01617	4.75E-08
0.03774	8.11E-08
0.0445	1.17E-07
0.0809	1.54E-07
0.1024	2.03E-07
0.1995	3.62E-07
0.4000	6.94E-07
0.6038	1.20E-06
0.8087	1.39E-06

Figure 17: Applied potential of + 0.2 V (vs. Ag/AgCl, KCl sat.); NADPH solutions prepared in PBS buffer 0.1 M, pH 7.4 between **1.01 mM** and **10.74 mM**; flow rate: 1.4 ml/min.

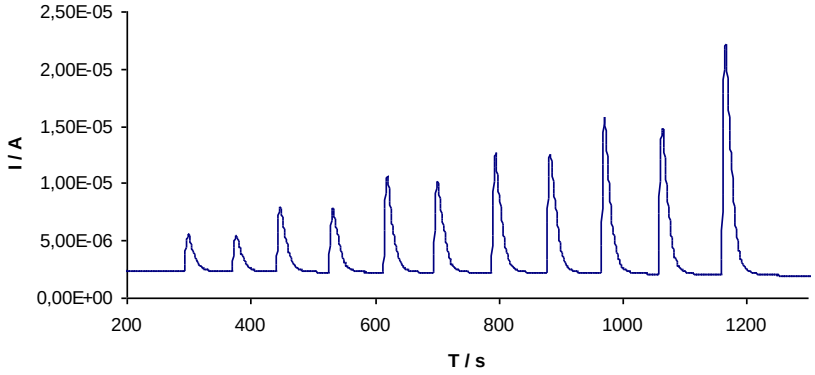


Figure 18: Calibration curve of NADPH measured by amperometry (FIA).

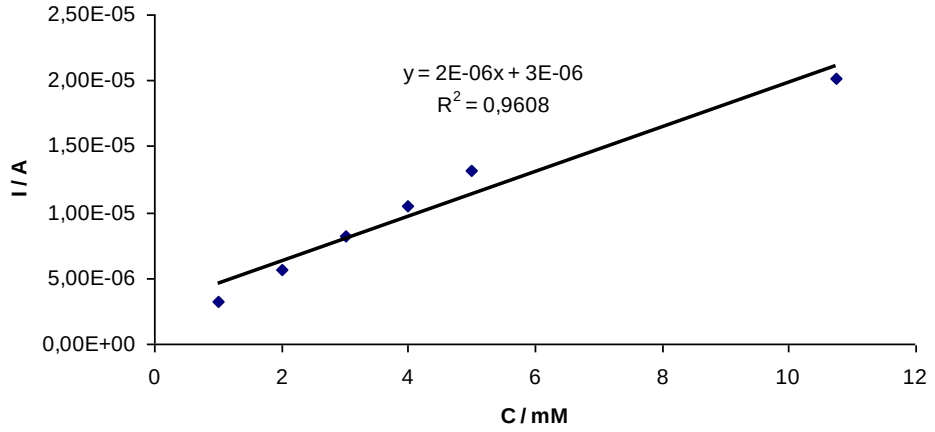


Table 5: Values of measured current representing the calibration curve (Fig. 18).

[NADPH]	Current media
C (mM)	I (A)
1.01	3.24E-06
1.998	5.65E-06
3.007	8.18E-06
3.995	1.04E-05
5.005	1.32E-05
10.74	2.01E-05

Amperometric calibration with mixed enzyme, $NADP^+$ cofactor and substrate solutions (FIA)

Calibration was carried out with solutions containing L-glutamate dehydrogenase (0.2056 units/ml), $NADP^+$ (1 mM) and L-glutamic acid solutions (between 9.18 x 10^{-2} mM and 3.9984 mM). All solutions were prepared inPBS buffer 0.1 M, pH 8.90. Measuring was performed at flow rate 1.4 ml/min.

The second part of FIA study was focused on the use of all reaction components. The enzyme was involved in the solution directly, and not immobilised in the electrode surface. Although the MG modified electrode is less sensitive to L-glutamic acid solution than to NADPH, we could observed that the reaction appeared, even if slowly. The oxidating process of NADPH, formed in the solution, was occuring. The current response was proportional to L-glutamic acid concetration.

From several pH media, pH 8.9 (PBS buffer) was chosen for the most obvious response.

Following figures show the amperometric behaviour of the solutions containing enzyme, $NADP^+$ and L-glutamic acid (Fig. 19) and calibration curve made consequently (Fig. 20).

Figure 19: Applied potential of + 0.2 V vs. Ag/AgCl, KCl sat; solutions prepared in PBS buffer 0.1 M, pH 8.90; L-glutamic acid solutions prepared between 0.0918 mM and 3.9984 mM mM; flow rate: 1.4 ml / min.

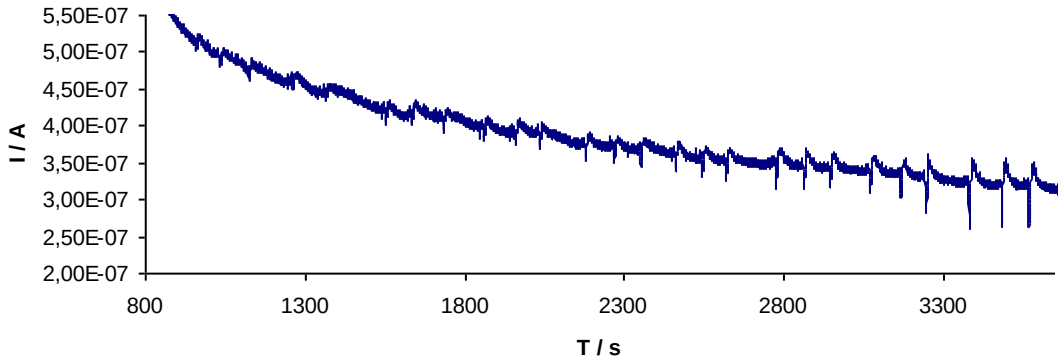


Figure 20: Calibration curve of mixed enzyme, cofactor $NADP^+$ and substrate solutions measured by amperometry (FIA).

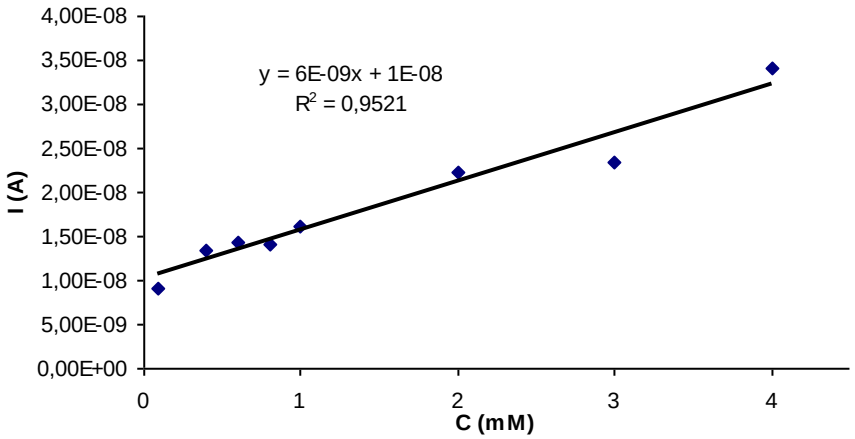


Table 6: Values of measured current representing the calibration curve (Fig. 20).

[L-GT]	Current media
C (mM)	I (A)
0.0918	9.18E-09
0.3978	1.35E-08
0.6018	1.44E-08
0.8058	1.40E-08
0.996	1.62E-08
1.9992	2.24E-08
2.9988	2.34E-08
3.9984	3.41E-08

Similar experiments performed with the enzyme immobilised in the sol-gel film, although mantainig the $NADP^+$ in the buffer used as carrier solution, revealed similar results. This behaviour leads us to conclude that at least in a first atempt it will be possible to get the proposed biosensor. However, further efforts are needed regarding optimisation either of chemical conditions as well the flow hydrodynamic parameters, aiming to improve the biosensor sensitivity and to exploit its application in real sample analysis.

5 CONCLUSION

We managed to construct working graphite electrodes modified with methylene green (MG) electropolymerization. This electrode was ment for creating of L-glutamate biosensor.

At first, assays of NADPH calibration were needed since a reduced form of $NADP^+$/NADPH coenzyme occurs as a reaction product and was intended to be oxidised by MG. The NADPH studies were implemented in a batch and a flow-injection system by voltammetry and amperometry, respectively.

We succeeded in reducing the potential for $NADP^+$ regeneration. A catalytic current could be observed for the NADPH oxidation at a lower potential (+ 0.2 V vs. Ag/AgCl, KCl sat.). The electrocatalytic current was proportional to the NADPH concentration and a good linear relation up to 8.62 mM was obtained. This demonstrates that MG is an effective mediator for the NADPH oxidation.

Afterwards, the electrodes were tested for L-glutamic acid detection and determination with the help of a L-glutamate dehydrogenase. This catalytic enzyme was presented either in sample solutions or immobilised in the layer of sol-gel glass. The enzyme FIA studies demonstrated that the modified electrode (with MG film) was less sensitive towards L-glutamic acid than to NADPH, probably due to the reversible behaviour of this enzyme.

Sol-gel encapsulation of the enzyme over the poly(methylene green) modified electrode proved to be effective but further studies (including optimisation of chemical conditions and the flow hydrodynamic parameters) must be made to reach the final biosensor and its application in real sample analysis.

6 LIST OF USED ABBREVIATIONS

FAD – flavin adenine dinucleotide

FAO - Food and Agriculture Organization

FDA - Food and Drug Administration

FIA - flow injection analysis

GDC - glutamate decarboxylase

GDH - glutamate dehydrogenase

GluOx - glutamate oxidase

MG - methylene green

MSG - monosodium glutamate

$NAD(P)^+$ - nicotinamide adenine dinucleotide (phosphate)

NAD(P)H – nicotinamide adenine dinucleotide (phosphate), reduced form

Ox – oxidised form

PBS - phosphate-buffered saline

PGS - poly(glyceryl silicate)

PMS - poly(methyl silicate)

Red – reduced form

SCE – saturated calomel electrode

SIA - sequential-injection analysis

TEOS - tetraethyl orthosilicate

WHO – World Health Organization

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8 SOUHRN

8.1 ÚVOD

Používání senzorů se stalo samozřejmostí našeho každodenního života. Vždyť samotné lidské smysly jako zrak, čich, sluch, chuť a hmat představují základní senzory lidského těla. Stejně tak automatické je pro nás např. laboratorní měření pH pomocí lakmusového papírku nebo pH-metru, v obou případech se opět jedná o použití senzorů. Různé typy čidel lze využít souhrnně k měření jakékoli energie (senzory elektrické, mechanické, optické, akustické, tepelné).

Ke správné činnosti chemického senzoru je zapotřebí zvolit vhodný citlivý prvek, který by selektivně reagoval na konkrétní chemickou látku. Jednou z variant je využití enzymů jako součást měřícího zařízení, neboť enzymy se pro svou vysokou selektivitu a citlivost ukázaly jako velmi praktické prostředky v analýze sloučenin. Bohužel enzymy, resp. jejich roztoky, mají některé nežádoucí vlastnosti, kvůli kterým je jejich použití omezené. Ve vodných roztocích podléhají vzdušné oxidaci, jejich terciární struktura bývá poškozena na fázovém rozhraní voda-vzduch, a tím klesá jejich katalytická aktivita. I tyto problémy však lze řešit, a to uzavřením (imobilizací) enzymu do alternativního prostředí, které jej stabilizuje a zachovává tak jeho aktivitu.

Mezi metody imobilizace patří fyzikální a chemická adsorpce, kovalentní vazba, cross-linking, jehož podstatou jsou intermolekulární vazby, a nebo zachycení v síti polymeru, tzv. entrapment.

Mnohé studie prokázaly výhody použití křemičitého skla v technice zachycování molekul (ale i celých buněk) do inertního materiálu, jenž tyto molekuly imobilizuje, ale zároveň v podstatné míře nenarušuje jejich vlastnosti. Křemičité sklo se vyrábí procesem sol-gel, při kterém se za nízké teploty hydrolýzou alkoxidových prekurzorů a následnou kopolymerací hydroxylovaných monomerů tvoří alkoxysilany, z nichž v kyselém prostředí vzniká pevná síť, materiál strukturou podobný obyčejnému sklu.

Jedním z hlavních aplikací sol-gelové techniky je výroba biosenzorů, kdy biosenzitivní materál (např. enzym) je přichycen pomocí sol-gelového skla k povrchu transduktoru.

Výběr transdukční metody určuje typ biosenzoru, ve většině případů se volí biosenzory optické nebo elektrochemické (amperometrické, potenciometrické). Výhodnými

vlastnostmi biosenzorů jsou spolehlivost, vysoká citlivost, přesnost a relativně nízká cena ve srovnání s dalšími detekčními technikami.

8.2 CÍL PRÁCE

Svou diplomovou práci jsem vypracovala na katedře fyzikální a analytické chemie Farmaceutické fakulty Univerzity v Portu v Portugalsku, pod programem Socrates/ Erasmus.

Podílela jsem se na vývoji elektrochemického biosenzoru pro stanovení L-glutamátu. L-glutamát sodný monohydrát je krystalická forma sodné soli kyseliny L-glutamové, jedné z přirozených aminokyselin obsažených v živých organismech. Používá se v potravinářském průmyslu jako přídatná látka pro zvýšení intenzity chuti pokrmu. V Evropské unii je její přítomnost v potravinářských výrobcích povinně uváděna kódem E 621. V USA je označován zkratkou MSG (*Mono Sodium Glutamate*). V minulosti panovaly obavy z nežádoucích účinků glutamanu sodného na lidský organismus. Byly popsány projevy častějšího užívání (zejména u citlivějších osob): jako zvracení, ztuhlé svaly, bušení srdce, závratě a bolesti hlavy, u astmatiků zhoršení jejich potíží. Upozorňovalo se i na možnou souvislost s Parkinsonovou a Alzheimerovou nemocí.

Rozsáhlá studie, uskutečněná pod záštitou Světové zdravotnické organizace (WHO) a Světové organizace pro zemědělství (FAO) (1987), však závažný vliv glutamátu na lidské zdraví nepotvrdila a proto ani nestanovila bezpečnou horní hranici denní dávky jeho příjmu v potravě. Bylo publikováno, že zcela bezproblémová hranice je 120 mg/kg hmotnosti člověka (mimo přirozený přísun v neupravených potravinách) a že denní příjem by neměl přesáhnout 10.5 g. Je však jen otázkou času, kdy vědci začnou znovu polemizovat o potřebě monitorování množství glutamátu v potravinách, případně o povinnosti výrobců uvádět jeho kvantitu na obalech výrobků. V takovém případě bude nutné najít efektivní, rychlou a levnou metodu stanovení L-glutamátu v produktech. Elektrochemický biosenzor je pro tento účel jednou z možných variant.

Glutamátový biosenzor, představený v této práci, byl připraven s použitím enzymu, glutamát dehydrogenázy, zachyceného ve vrstvě sol-gelového skla na povrchu grafitové

elektrody. Transdukce signálu enzymové aktivity byla vedena elektrochemickou cestou (voltametricky a amperometricky). L-glutamát dehydrogenáza vyžaduje ke své činnosti přítomnost nikotinamidového koenzymu ($NAD(P)^+$/ NAD(P)H), jehož redukovanou formu (produkt reakce) je výhodné regenerovat. K šetrné oxidaci NADPH za sníženého napětí bylo zvoleno ve vodě rozpustné barvivo – methylenová zeleň, jejíž tenký film na povrchu elektrody byl vytvořen pomocí elektropolymerizace.
Mezi hlavní metody hodnocení funkčnosti celého systému patřila cyklická voltametrie a průtoková injekční analýza (FIA) s amperometrickou detekcí.

8.3 VÝSLEDKY A ZÁVĚR

Počáteční spektrofotometrická studie prokázala, že průběh oxidace substrátu (L-glutamátu) není negativně ovlivněn přítomností methylenové zeleně (MG, *methylene green*) a že použití MG jako mediátoru pro regeneraci NADPH je uskutečnitelné a efektivní. Chování reakční směsi (L-glutamát, L-glutamát dehydrogenáza, $NADP^+$ a MG) vystihuje absorpční křivka na Obr. 10.
Během následujícího cyklovoltametrického měření v neprůtokovém systému (skleněná cela) jsme prověřili citlivost vyrobené grafitové elektrody, upravené MG elektropolymerizací, ke koenzymu reakce. Pozorovali jsme oxidaci NADPH za sníženého napětí (+ 0.2 V vs. Ag/AgCl, referenční elektroda saturovaná KCl) a lineární závislost elektrokatalytického proudu na koncentraci NADPH (0.1 – 8.62 mM). Cyklovoltamogram a kalibrační křivka jsou uvedeny na Obr. 13 a 14.
Stejný detektor jsme poté zapojili do systému průtokové injekční analýzy (FIA) a odpověď senzoru na zvyšující se koncentraci NADPH měřili amperometricky při + 0.2 V vs. Ag/AgCl (KCl sat.). Intenzita píků byla přímoúměrná koncentračnímu rozmezí 2.696×10^{-3} mM – 10.74 mM (viz. Obr. 15 – 18).
Cílem posledních pokusů této práce bylo vyzkoušet senzor pro detekci a stanovení L-glutamátu s pomocí katalytického enzymu. L-glutamát dehydrogenáza byla přítomna ve zkoušeném roztoku, anebo imobilizována ve vrstvě křemičitého skla na povrchu elektrody. Uspokojivých výsledků bylo (za mé pracovní přítomnosti v Portu) dosaženo jen v prvém případě. Zjistili jsme, že grafitová elektroda modifikovaná MG je citlivá k přítomnosti L-glutamátu v různých koncentracích (Obr. 19 a 20), i když v menší míře

než tomu bylo u NADPH. Výsledek mohl být zkreslen reversibilním chováním enzymu. Pracovní tým (po mém odjezdu) dále dosáhl podobných výsledků i s použitím techniky imobilizace enzymu.

Uchycení enzymu do vrstvy sol-gelu, a tedy konstrukce biosenzoru, se ukázalo být proveditelné a fungující, je však nutno provést další studie (včetně optimalizace podmínek měření) k dosažení efektivních výsledků.